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# *In vitro* stimulation of lymphocytes with an antigen fraction prepared from *Mycobacterium leprae* and tuberculin PPD in contacts and non-contacts of leprosy patients

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## SUMMARY

An antigen fraction from *Mycobacterium leprae*, called MLW1, was used as stimulator in the lymphocyte stimulation test, for comparison with tuberculin PPD. The test was performed in three groups of contacts of leprosy patients with various degree of exposure: (1) close contacts, (2) healthy occupational contacts and (3) non-close contacts and, in addition, in a group of BCG vaccinated and non-exposed controls. The MLW1 preparation induced moderate to strong responses in all three groups of contacts. Although the close contact group showed the highest median responses to all three doses tested, there were no significant differences between the contact groups. At all three dose levels the non-exposed group showed markedly and significantly lower median responses than the contact groups. The responses to tuberculin PPD was markedly and significantly lower in the close contact group than in the other groups. Both when individual responses to the two antigens MLW1 and PPD are compared and when the $\Delta$ct/min′ estimator is used, the results indicate that the intensity of the specific response increases with the closeness of contact with leprosy patients.

**Keywords** lymphocyte stimulation *Mycobacterium leprae* tuberculin PPD leprosy

## INTRODUCTION

It has been claimed that the risk of acquiring leprosy increases with the duration and closeness of contact with leprosy patients (Badger, 1959; Doull *et al.*, 1942). Development of clinical disease, which only occurs in a small number of the infected subjects, is highly dependent on the individual's capacity to mount a cell-mediated immune response to *Mycobacterium leprae* (Godal, 1978).

By means of the lymphocyte transformation test, the responses were observed to increase with increasing exposure to leprosy patients (Godal, Løfgren & Negassi, 1972; Myrvang *et al.*, 1975). However, a higher number of non-responders among household contacts of untreated and shortly treated lepromatous leprosy (LL) patients than of tuberculoid (TT) and treated LL patients was found, suggesting that an increased risk of acquiring leprosy among contacts of LL patients may be related to a decrease of host resistance caused by 'superexposure' to *M. leprae* (Godal & Negassi, 1973).

In contrast, Menzel, Bjune & Kronvall (1979a) found that household contacts of LL patients, but not of TT patients, had significantly greater responses to *M. leprae* in the lymphocyte stimulation test (LST) than controls and that household contacts of highly bacilliferous LL patients

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had stronger responses than those of LL patients with a low bacillary load. Although there is a considerable specificity in the *in vitro* lymphocyte responses to antigens of *M. leprae* and *M. tuberculosis*, studies in non-exposed subjects have shown that the responses to *M. leprae* were related to the tuberculin reactivity of the test subject (Closs, 1975). In untreated LL patients with a specific lack of response to *M. leprae* antigens, we have earlier found that also their responses to tuberculin purified protein derivative (PPD) were decreased compared with healthy contacts of leprosy patients (Reitan, Closs & Belehu, 1982). This could indicate that their response to other mycobacteria may become affected because they contain antigens that cross-react with *M. leprae*.

In a previous paper we introduced (Closs *et al.*, 1982) the use of the ct/min′ estimator, which takes into account both the degree of cross-reactivity between the responses to a *M. leprae* antigen fraction (MLW1) and PPD and the magnitude of the MLW1 response. By using this estimator, we obtained a better discrimination of the responses between exposed and non-exposed individuals, and consequently an improved specificity was observed.

In the present study we have tested contacts of leprosy patients with various degrees of exposure to patients and compared them with non-exposed controls by measuring the *in vitro* lymphocyte responses to the *M. leprae* antigen preparation MLW1 and tuberculin PPD.

## MATERIALS AND METHODS

*Contacts.* Three groups of Ethiopians with various degrees of exposure to leprosy were included in this study. (1) Close contacts (CC), 14 individuals who were all family members of patients with LL, being either children or siblings of a patient, and who were living in the same household as the patient. The group consisted of seven males and seven females, and the median age was 11 years (range 7–30 years). Eight of them were BCG vaccinated. None of them had clinical signs of leprosy. (2) Healthy occupational contacts (HC), 17 individuals with occupational contact with leprosy patients, all of whom were staff members of the Armauer Hansen Research Institute (AHRI) or the All Africa Leprosy and Rehabilitation Training Centre (ALERT) in Addis Ababa. The median time of exposure was 6 years (range 5 months to 14 years). All were in regular contact with patients except for two who were office personnel. The group included 12 males and five females and the median age was 26 years (range 18–34 years). Thirteen of them were BCG vaccinated. Eight individuals from our previous study (Reitan *et al.*, 1982) were retested and included in the present study. (3) Non-close contacts (NCC), 15 individuals who had no family members with leprosy and were not in regular contact with leprosy patients. They were all, except for two of them, from Addis Ababa but not living near the area of the leprosy hospital. The group included seven males and eight females with a median age of 25 years (range 11–42 years), and only two of them were BCG vaccinated.

*Non-exposed controls (NEC).* Eighteen healthy, BCG vaccinated and tuberculin positive Norwegians who were hospital staff mainly and had not been exposed to leprosy or any *M. leprae* infected material, were included as a control group. There were eight males and 10 females, and the median age of this group was 28 years (range 20–42 years).

*Stimulants.* The following stimulants were used in the LST. (a) A fractionated preparation of *M. leprae* of armadillo origin, called MLW1, batch S30, prepared by the procedure of Closs *et al.* (1982), was shown to contain mainly *M. leprae* antigen 7 by crossed immunoelectrophoresis. (b) Tuberculin PPD, batch RT23 (a few tests were performed with a similar preparation batch RT38), obtained from Statens Seruminstitut, Copenhagen, Denmark. (c) Amoeba antigen, consisting of freeze dried organisms of washed *Entamoeba histolytica.* (d) Phytohaemagglutinin (PHA), HA15, were both obtained from Wellcome Reagents Limited, Beckenham, UK.

*Cultures of lymphocytes.* Mononuclear cells were isolated and cultured as described previously (Closs *et al.*, 1982). In brief, the cells, $10^5$/well, were cultured in triplicate in round bottom microtitre trays (ISMR 96TC, Linbro Chemical Co., New Haven, Connecticut, USA (for the contacts) or 3799, Costar, Cambridge, Massachusetts, USA (for the controls)) in 225 $\mu$l of RPMI 1640 medium (Flow Laboratories, Ayrshire, UK) containing 20% pooled human serum and supplemented with glutamine (2 mM) and antibiotics (100 u/ml penicillin and 100 $\mu$g/ml streptomycin). The antigen

stimulated cells were cultured for 6 days and the mitogen stimulated cells for 72 h at 37°C in 5% $CO_2$ in humidified air before being harvested. Each culture received 1 $\mu$Ci of (methyl-$^3$H)-thymidine (sp. act. 2·0 Ci/mmol, Radiochemical Centre, Amersham, UK) 18 h before harvest. The cells were harvested with a multiple harvester, placed onto glass fibre filters and washed in distilled water. Thymidine incorporation was measured in a liquid scintillation counter (SL30, Intertechnique, France). The median counts per minute (ct/min) of each triplicate was used, and the degree of stimulation expressed as Δct/min = ct/min of stimulated triplicate − ct/min of unstimulated control culture. The Δct/min′ estimator was calculated from the formula:

$$\Delta ct/min' = \frac{\Delta ct/min_{MLW1}}{\Delta ct/min_{PPD}} \times \Delta ct/min_{MLW1}.$$

The calculation was based on the stimulation obtained with the final concentration in the culture of 0·1 $\mu$g/ml for both MLW1 and PPD. To avoid the problems connected with very low values of Δct/min, all responses of less than 500 Δct/min were given the value of 500.

*Statistical methods.* Wilcoxon's rank sum test (Diem, 1962) was used to compare groups of subjects, and $P < 0{\cdot}05$ was set as the limit for statistical significance.

## RESULTS

*In vitro* lymphocyte responses to MLW1 and tuberculin PPD were studied in three groups of contacts of leprosy patients (the CC group, the HC group and the NCC group) and in addition, in the control group NEC. Fig. 1 shows the LST responses to three doses of MLW1 (0·01, 0·1, and 1·0 $\mu$g/ml) which refer to final concentrations in the cultures. A wide variation in the responses was seen in all three groups of contacts. Although the CC group showed the highest median responses to all three doses, there were no significant differences between the three groups of contacts. An individual

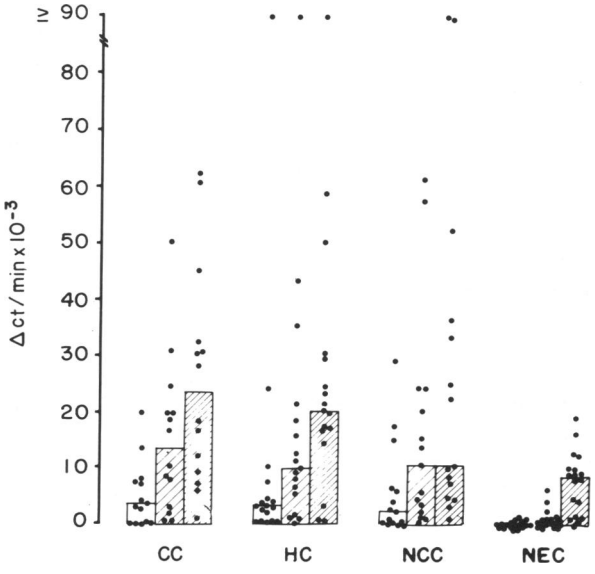


**Fig. 1.** *In vitro* lymphocyte stimulation with the MLW1 antigen fraction prepared from *M. leprae*, at three different concentrations: 0·01 $\mu$g/ml (□), 0·1 $\mu$g/ml (■) and 1·0 $\mu$g/ml (▨), using peripheral blood lymphocytes from groups of contacts of leprosy patients: CC ($n = 14$); HC ($n = 17$); NCC ($n = 15$) and NEC ($n = 18$). Each point represents one individual, and the height of each bar corresponds to the median of that group. The responses are shown as net counts (Δct/min).

who showed a response with Δct/min higher than the mean background level of unstimulated cultures for all individuals included (i.e. 1,737 ± 147 ct/min) was defined as a responder. In the NEC group there were three responders to 0·1 μg/ml and 13 to 1·0 μg/ml. But to all three doses the median responses in the NEC group were markedly and significantly lower than in any of the contact groups. The dose of 0·1 μg/ml appeared to give the best discrimination between exposed and non-exposed individuals, since it induced fairly strong responses in contacts and a relatively low number both of responders in the non-exposed and of non-responders in the exposed individuals. There were three individuals in the CC group, four in the HC group and three in the NEC group who were non-responders, i.e. their stimulated cultures were found to generate 1,737 Δct/min or less.

The responses to tuberculin PPD in the same groups of individuals are shown in Fig. 2. Although a wide variation in the responses was observed in all groups, the median responses in the CC group were markedly lower than in any of the other groups to all three doses, and the differences in the responses were significant for the HC group to all three doses and for the NCC and NEC groups to the two highest doses. The optimal dose for PPD seemed to be 1·0 μg/ml for all groups included.

In Fig. 1 minimal responses were seen to 0·1 μg/ml of MLW1 in the NEC group, while most of the individuals showed moderately strong responses to 1·0 μg/ml of the antigen. The four individuals with the highest responses to 1·0 μg/ml with Δct/min > 10,000, showed also strong responses to PPD, being above the median response at each dose. This indicates that cross-reacting determinants on the MLW1 antigen contributed to the relatively strong responses to this dose.

As can be seen in Table 1, there was no depression of the responses to the non-specific mitogen PHA and the non-mycobacterial amoeba antigen in the CC group compared with the other contact groups, showing no evidence of a non-specific depression of the immune response.

The age distribution in the CC group differed from the other groups by consisting of a relatively high proportion of pre-pubertal individuals. We therefore wanted to know whether this disproportion could have influenced the results of this group. Table 2 shows that the median response to MLW1 in the individuals below 13 years of age is lower than in those above this age, whereas in the responses to PPD it is opposite, at both 0·1 and 1·0 μg/ml (data not shown). These results show that the pre-pubertal responses did not contribute to the markedly lower PPD responses in the CC group compared with the other groups. Comparison of the PPD responses

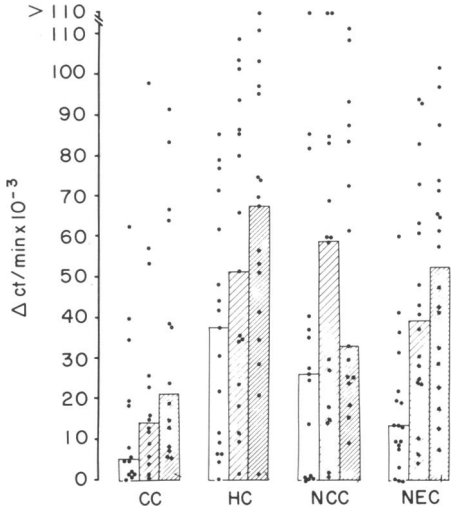


**Fig. 2.** *In vitro* lymphocyte stimulation with tuberculin PPD at three different concentrations: 0·1 μg/ml (□), 1·0 μg/ml (■) and 10 μg/ml (▨). For further explanation, see legend to Fig. 1.

**Table 1.** *In vitro* lymphocyte stimulations with PHA and *E. histolytica* in the various groups of contacts of leprosy patients

| Groups of individuals | No. | Δct/min (mean ± s.d.) PHA* | *E. histolytica*† |
|---|---|---|---|
| Close contacts | 14 | 121,100 ±61,100 | 16,300 ±12,100 |
| Healthy occupational contacts | 17 | 101,300 ±29,500 | 15,800 ±20,500 |
| Non-close contacts | 15 | 105,100 ±52,700 | 13,700 ±16,900 |

* 1:100 dilution of stock; † $4 \times 10^3$ parasites/ml.

**Table 2.** *In vitro* lymphocyte stimulations presented as median values of nine individuals less than 13 years and of five individuals more than 13 years old belonging to the close contact group

| Age group | No. | Δct/min MLW1* | PPD* |
|---|---|---|---|
| Below 13 years | 9 | 10,100 | 18,300 |
| Above 13 years | 5 | 16,700 | 4,500 |
| Total | 14 | 13,400 | 5,100 |

* Dose = 0·1 μg/ml.

between BCG vaccinated and non-vaccinated individuals showed no significant differences either when only the CC group or when all the contacts were included.

The responses of each individual to optimal doses of the two cross-reacting antigens MLW1 (0·1 μg/ml) and PPD (1·0 μg/ml) are directly compared in Fig. 3. All the 18 individuals of the NEC group showed a response to PPD which was at least 10 times higher than to MLW1, whereas this was found in only two out of 14 individuals in the CC group, in four out of 17 in the HC group and in four out of 15 in the NCC group. Thirteen of the HC group and 10 of the NCC group showed a response to PPD which was three times higher than to MLW1, whereas this was observed in only three individuals of the CC group. In contrast to the NEC group, which was exposed to only one of the antigens, a group exposed only to *M. leprae* is lacking in the comparison. By multiplying the ratio between the LST responses to MLW1 and PPD with the response to MLW1, an estimator (Δct/min′) is obtained which adjusts for exposure to *M. tuberculosis*/BCG and therefore can be used as an indicator of exposure in populations where sensitization to both antigens occurs. Fig. 4 shows the Δct/min′ responses to 0·1 μg/ml of both MLW1 and PPD. The variations are wide in all four groups, but the median response in the CC group is ten times as high as in the HC and NCC groups, the difference being significant even for the HC group ($P < 0{\cdot}05$). Compared with the NEC group, the median response in the CC group is 1,000 times and in the HC and NCC groups 100 times as high as in the NEC group, which is highly significant ($P < 0{\cdot}005$). A complete dissociation of the Δct/min′ responses was not found between the groups of contacts and the non-exposed controls in the present study. But the overlap region consisted mainly of the non-responders to MLW1 in the contact groups and of the responders to MLW1 in the non-exposed group. Both when the individual responses to the two antigens MLW1 and PPD are compared, as in Fig. 3, and when the Δct/min′ estimator is used, as in Fig. 4, we found that the intensity of the specific response increases with the closeness of contact with patients.

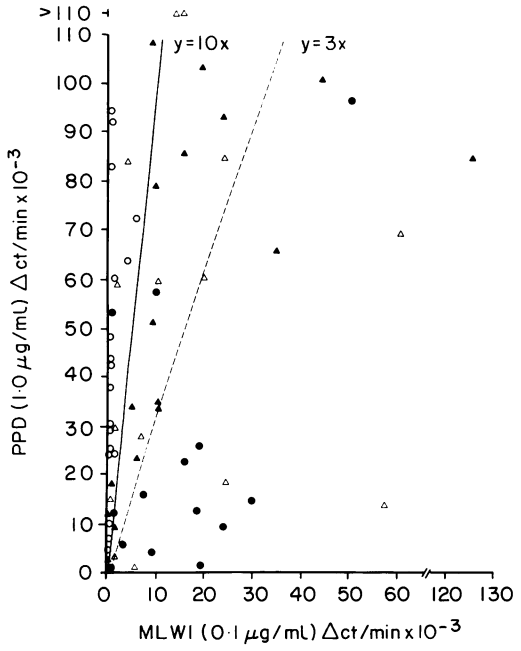


**Fig. 3.** *In vitro* lymphocyte stimulation of lymphocytes in contacts of leprosy patients: CC, $n = 14$ (●); HC $n = 17$ (▲); NCC, $n = 15$ (△) and NEC, $n = 18$ (○), with MLW1 and tuberculin PPD. The lines drawn are $y = 3x$ and $y = 10x$. For further explanation see legend to Fig. 1.

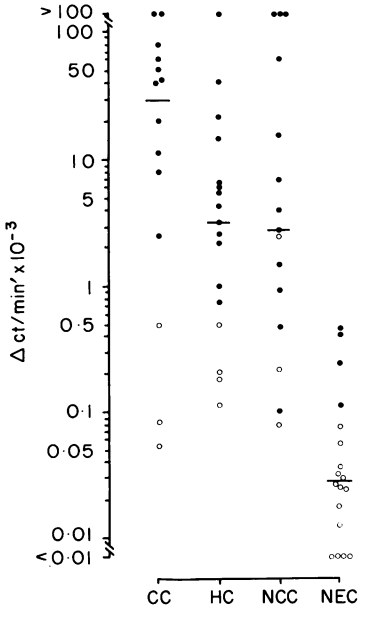


**Fig. 4.** *In vitro* lymphocyte stimulation with MLW1 (0·1 μg/ml) and tuberculin PPD (0·1 μg/ml) in contacts of leprosy patients: CC, $n = 14$, HC, $n = 17$; NCC, $n = 15$ and NEC, $n = 18$. The horizontal lines indicate the median of each group. Stimulation is shown as $\Delta ct/min' = \Delta ct/min_{MLW1}{}^2/\Delta ct/min_{PPD}$. Non-responders to MLW1 (i.e. $\Delta ct/min < 1{,}737$) are indicated with open circles (○).

## DISCUSSION

There is a close antigenic relationship between *M. leprae* and *M. tuberculosis* and other mycobacterial species (Harboe *et al.*, 1977). The immune response mounted in an individual exposed to *M. leprae* therefore consists of both a specific and a cross-reacting component. In untreated LL patients with a specific lack of response to *M. leprae* antigen, we showed (Reitan *et al.*, 1982) a depression in the responsiveness to the cross-reacting antigen tuberculin PPD, indicating that the response to cross-reacting determinants on the antigen was affected by the specific non-responsiveness to *M. leprae*. In the present study we have compared the responses to these two antigens in contacts of leprosy patients with various degree of exposure. Although the group of close contacts showed the highest median response to MLW1, there were no significant differences between the contact groups.

In contrast to Menzel *et al.* (1979a), who showed stronger *M. leprae* responses in household contacts of LL patients than in controls, Godal & Negassi (1973) found a higher number of non-responders among contacts of untreated LL patients than treated patients, suggesting that an increased risk of acquiring leprosy among contacts of LL patients may be related to a decrease of host resistance caused by 'superexposure' to *M. leprae*. In our results, it may be that both these reported phenomena are operating in the MLW1 responses in the CC group. However the median response to tuberculin PPD was markedly and significantly depressed in the CC group compared with the other contact groups. Thus the pattern of response to PPD in the CC group has some similarities with that of the untreated LL patients from our previous study (Reitan *et al.*, 1982). Since this CC group showed the highest median responses both to PHA and the amoeba antigen, there is no indication of a non-specific non-responsiveness in that group.

The demonstration of a decreased responsiveness to PPD in CC is of the greatest interest. Because of the heavy exposure to *M. leprae* in that particular group of contacts, it might be that their ability to respond to those determinants of PPD which are shared with *M. leprae* is reduced. To elucidate whether this decrease in the PPD responses is an indicator for a high risk individual or even for a subclinical infection, will be important. It has been observed that the percentage of specific positive tuberculin reactions in patients who recently developed high resistant TT leprosy was lower than in healthy people of the same age and living in the same area (Leiker, 1960), and that a much lower rate of conversion to tuberculin positivity after vaccination with a vole tuberculosis vaccine was observed in children from families with leprosy than in children from families with no history of leprosy (Jamison & Vollum, 1968).

Menzel *et al.* (1979b), reported that sensitization to *M. leprae* antigens was present in a high number of the 6–14 year old household contacts of active lepromatous patients and concluded that opportunity for exposure rather than age in itself determines the age specific findings. Our results are in agreement with theirs since our CC group which consists of a high number of pre-pubertal individuals, showed median responses to MLW1 at all three doses tested which were higher than those of the other contact groups. Stanford, Shield & Rook (1981) reported that both increasing age and BCG vaccination increase the percentage of children responding to mycobacterial species present in their environment. It was therefore reasonable to expect that the marked decrease in the PPD responses in our CC group could be explained by that. But in our study the median PPD response of the nine individuals less than 13 years old was higher than that of the five above this age. Accordingly, a lower median $\Delta$ct/min′ response in the group less than 13 years old, $\Delta$ct/min′ = 11,500, than in the one more than 13 years old, $\Delta$ct/min′ = 60,800, was observed. These results show that the pre-pubertal responses did not contribute either to the somewhat lower PPD responses or to the somewhat higher median $\Delta$ct/min′ responses in the CC group compared with the other groups. Similarly comparison between the PPD responses of the frequency of BCG vaccinated and non-vaccinated individuals of this group showed that BCG vaccination did not influence the results.

In a study of the specific LST responses to *M. leprae* a group of individuals exposed only to *M. leprae* and not to *M. tuberculosis* should have been included. Such a group is lacking in the present study, because it represents a very rare epidemiological situation. Furthermore, the NEC group and

the groups of contacts are exposed to very different spectra of environmental mycobacterial species. Various ways of presenting the data from the LST have been reported, i.e. net increment in counts, stimulation index, relative proliferative index (Dean *et al.*, 1977), and moles uptake of nucleoprotein base per unit number of lymphocytes (Burford-Mason & Gyte, 1979). To express specific sensitization to *M. leprae* in individuals exposed both to *M. leprae* and *M. tuberculosis*, without having a specific antigen, we have previously introduced the use of the Δct/min′ estimator (Closs *et al.*, 1982), which takes into account both the ratio between the responses to MLW1 and the cross-reacting antigen PPD and the intensity of the MLW1 response. By using this estimator, we obtained a better discrimination of the responses between exposed and non-exposed individuals. In the present study we have applied this estimator in the study of the LST responses in groups of contacts of leprosy patients. In contrast to the previous work where the Δct/min′ responses were based on optimal doses of the two antigens, 0·1 μg/ml of MLW1 and 1·0 μg/ml of PPD, the responses in the present work are based on equal doses (0·1 μg/ml) of both antigens in order to get responses of comparable magnitude and a minimal influence of cross-reacting determinants. Since some non-responders in the various groups has to be expected, the Δct/min′ estimator will not always show a complete discrimination between exposed and non-exposed when individuals are compared. The present results show that as long as a truly specific antigen reagent is lacking, Δct/min′ can enable us to discriminate between groups of individuals with a differing exposure to *M. leprae*.

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